nitropropane. A trace of N-(2-nitroisobutyl)-piperidine was obtained by means of an acid catalyzed amine exchange.

This investigation has revealed no exception to the proposal that alkylation of nitroparaffins by amines must proceed through an elimination–addition mechanism.

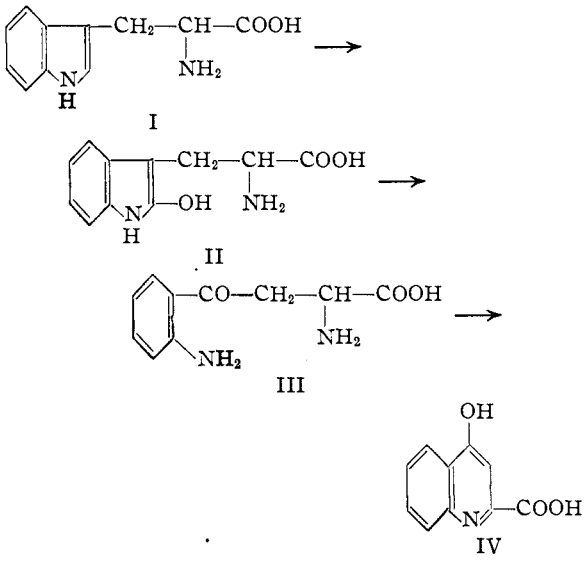
URBANA, ILLINOIS RECEIVED MARCH 16, 1950

[CONTRIBUTION FROM THE INSTITUTE OF POLYTECHNICS, UNIVERSITY OF CITY OSAKA]

Studies on Amino Acids. III. The Synthesis of α-Hydroxytryptophan

BY MUNIO KOTAKE, TAKEO SAKAN AND TOSHIO MIWA

Y. Kotake,[1] *et al.*, have demonstrated the presence of kynurenine, as well as kynurenic acid, in the urine of rabbits which have been fed tryptophan. They have postulated the following sequence of reactions in the intermediary metabolism of tryptophan.



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α-Hydroxytryptophan, which is postulated as an intermediary in the above scheme, was isolated by Wieland and Witkop[2] from the hydrolysis product of phalloidin, a toxic principle found in a poisonous mushroom, *Amanita phalloides*. The yield of amino acid was too small to permit biological experiments. Later, Butenandt[3] was able to show that this compound possesses a hereditary-controlling gene hormone activity, though weaker than kynurenine, in producing the color of eyes of *Drosophila melanogaster;* in this respect α-hydroxytryptophan acts as pro-kynurenine.

Previous attempts to synthesize α-hydroxytryptophan proved unsuccessful.[4,5] The optically active α-hydroxytryptophan which has been obtained by oxidizing L-tryptophan with peracetic acid under controlled conditions is proved, recently, by Witkop[6] to be identical with that obtained from phalloidin.

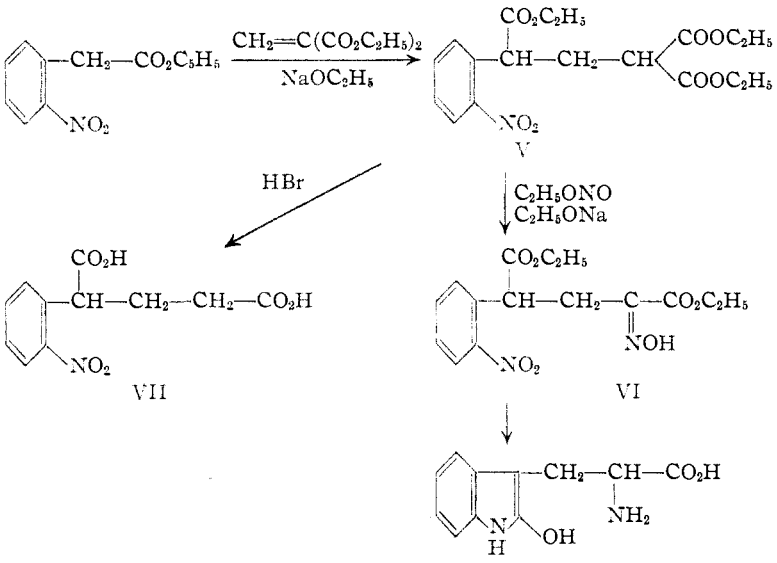
In our investigations, we found that *o*-nitrophenylacetic acid ethyl ester undergoes the Michael condensation with diethyl methylenemalonate in the presence of sodium ethoxide. The product was treated with 48% hydrobromic acid and decarboxylation took place, giving an acid compound with formula $C_{11}H_{11}O_6N$. When the condensation product was treated with ethyl nitrite and sodium ethoxide, a corresponding oximino ester was produced with 60% yield.

Since *o*-nitrophenylsuccinic acid was obtained by hydrolyzing this ester with hydrochloric acid, the oximino ester must have the structural formula (VI) and thus the chemical structure of the condensation product must be (V). The acid with the empirical formula $C_{11}H_{11}O_6N$ is therefore shown to be *o*-nitrophenylglutaric acid (VII).

On reducing the oximino ester (VI) at 0° with stannous chloride and hydrochloric acid in glacial acetic acid, the amino ester crystallized out with tin chloride as a double salt. After removing the tin with hydrogen sulfide, the product was hydrolyzed with hydrochloric acid and concentrated in an atmosphere of carbon dioxide. The hydrochloride of the amino acid was obtained in a crystalline form, which after recrystallization from water decomposes at 208°. The free amino acid, decomposition point 248–249°, was obtained from this hydrochloride.

The analytical results obtained for this compound closely agree with the data calculated for α-hydroxytryptophan and furthermore a violet color shown by the ninhydrin test characterizes the compound as an α-amino acid. In the Millon and Folin–Denis reactions the compound gave a yellowish-orange and a dark blue color, respectively. An aromatic amine, which was liberated only when the amino acid was warmed with alkali, could be diazotized and coupled with β-naphthol to produce a dye. The positive "Pine splint reaction" was shown when the amino acid was dry distilled with zinc dust. These facts indicate the presence of oxindole residue in the molecule. The results obtained by these tests agree with those reported by Wieland for the α-hydroxytryptophan, which has been isolated from phalloidin. The amino acid we have

(1) Y. Kotake, *Z. physiol. Chem.*, **195**, 158 (1931).
(2) Wieland and Witkop, *Ann.*, **543**, 171 (1940).
(3) Butenandt, *Naturwiss.*, **28**, 447 (1940).
(4) Fischer and Smeykal, *Ber.*, **56**, 2368 (1923).
(5) Julian, Pikl and Wantz, THIS JOURNAL, **57**, 2026 (1935).
(6) Witkop, *Ann.*, **558**, 98 (1947).



obtained here, therefore, proves to be DL-α-hydroxytryptophan. Experiments are now being conducted to resolve the amino acid into its optically active isomers.

Experimental

1-(*o*-Nitrophenyl)-propane-1,3,3-tricarboxylic Acid Triethylester (V).—To a solution of 2.3 g. of sodium dissolved in 60 cc. of anhydrous alcohol was added a warm solution of 20.9 g. (0.1 mole) of *o*-nitrophenylacetic ester (m. p. 64°) dissolved in 300 cc. of anhydrous alcohol. The mixture, which develops a dark blue color, was cooled to −8° and to it was added with stirring 36 g. (0.2 mole) of freshly distilled diethyl methylenemalonate in 40 cc. of anhydrous alcohol at a temperature which does not exceed 0–2°. After the addition was completed, the reaction mixture was brought to room temperature. The stirring was continued for twenty-four hours. The solution at this stage showed a red color. Dry carbon dioxide was passed through the reaction mixture and the alcohol removed under reduced pressure. The residue was extracted with ether, the ether solution dried over anhydrous sodium sulfate, and the solvent removed. An orange-red colored oil, thus obtained, was distilled *in vacuo;* up to 170° (0.015 mm.) *o*-nitrophenylacetic ester came over and the condensed product (V), a yellowish red oil, distilled over at 170–180° (0.015 mm.); yield 60%.

Seven cc. of 48% hydrobromic acid was added to 1.0 g. of this product and the mixture boiled for one hour. It was then concentrated after the addition of some water. A crystalline product deposited which was purified by recrystallization from water; m. p. 150–150.5° with decomposition. The analytical results shown below (the samples used were dried at 110° for two hours) closely agree with the data calculated for *o*-nitrophenylglutaric acid.

Anal. Calcd. for $C_{11}H_{11}O_6N$: C, 52.17; H, 4.38. Found: C, 52.41; H, 4.55.

1-(*o*-Nitrophenyl)-3-oximinoglutaric Acid Diethylester.—To a solution of 1.7 g. of sodium dissolved in 40 cc. of anhydrous alcohol, cooled to −10°, was added 28.5 g. of the ethyl ester of the tricarboxylic acid (V) in 20 cc. of anhydrous alcohol. Under constant stirring 11.2 g. of ethyl nitrite was added dropwise to this cooled mixture and after the completion of the addition the reaction mixture was stirred for fifteen hours at −10°. The solvent was then removed under reduced pressure, ice water added to the residue, and carbon dioxide gas passed through the aqueous solution. The solution was extracted with ether, the ether extract washed with water, dried over calcium chloride, and the solvent removed. When the sides of the container were rubbed with a glass rod, the residue became crystalline. Recrystallized from a mixture of benzene and petroleum ether the rhombic crystals melted at 92°. A yield of 62% was obtained.

Anal. Calcd. for $C_{15}H_{18}O_7N_2$: C, 53.25; H, 5.36; N, 8.28. Found: C, 53.27; H, 5.58; N, 8.39.

No appreciable change in character was shown when 0.2 g. of this sample was warmed on a water-bath with 3 cc. of 20% hydrochloric acid and 2 cc. of water. It went into solution, however, when heated with 2 cc. of 35% hydrochloric acid over an open flame. On concentrating the solution after diluting with water some crystalline material was obtained. It melted, after recrystallization from water, at 187° with decomposition and no depression was observed when its mixed melting point was taken with *o*-nitrophenylsuccinic acid.[7]

α-Hydroxytryptophan Hydrochloride (II).—Dry hydrogen chloride was passed at 0° through a mixture of 45 g. of stannous chloride in 95 g. of glacial acetic acid until a gain in weight of 20 g. was indicated. To this cooled mixture was added 6.8 g. of the oximino ester, in portions. The reaction mixture was placed at 0–2° for five days and the white precipitate which formed was collected and washed with glacial acetic acid. It was then dissolved in 150 cc. of water, saturated with hydrogen sulfide and the precipitate which formed was centrifuged. The precipitate was repeatedly extracted with warm water (50°), and the extracts were combined and concentrated below 50° under reduced pressure in a carbon dioxide atmosphere. When the volume of the residue was reduced to about 15 cc., an equal volume of 20% hydrochloric acid was added and the reaction mixture heated to 100° for one hour. The solution was again concentrated under reduced pressure to 10 cc. and diluted. When concentrated a third time, after the addition of 30 cc. of water, the entire content became a crystalline solid. White needles obtained after recrystallization from water decomposed at 208°; yield 58%.

Anal. Calcd. for $C_{11}H_{12}O_3N_2 \cdot HCl \cdot H_2O$: C, 48.09; H, 5.50; N, 10.20; loss in weight, 6.6. Found: C, 48.29; H, 5.66; N, 10.41; water of crystallization, 6.4.

The ninhydrin test, with the addition of a small amount of sodium acetate, gave a reddish purple color; the Millon reaction a yellowish orange color; and the Folin–Denis reaction, a dark blue color. A few mg. of the crystals were boiled for three min. with four drops of a 5% solution of baryta and with cooling diazotized by adding 1–2 mg. of sodium nitrite and 2 drops of 2 *N* hydrochloric acid. When 6 drops of a solution of 2–3 mg. of β-naphthol dissolved in 2 *N* sodium hydroxide was added to the aqueous mixture, a beautiful red color developed. If the treatment of the sample with baryta was omitted, no red colored dye was produced.

α-Hydroxytryptophan.—To α-hydroxytryptophan hydrochloride dissolved in water is added a warm aqueous solution of silver sulfate. The silver chloride which precipitated was filtered off, and the excess silver removed as silver sulfide by passing hydrogen sulfide through the filtrate. The clear solution was concentrated to one-third of its volume under reduced pressure. It was then neutralized with barium hydroxide to remove the sulfuric acid present, and at 40° concentrated to a sirupy consistency in an atmosphere of nitrogen. A small amount of water was added and by warming on the water-bath and rubbing, the material became solid. After treating the solid mass with a

(7) Fr. Fichter and O. Walter, *Ber.*, **42**, 4312 (1909).

large quantity of water and concentrating it *in vacuo* in an atmosphere of carbon dioxide, the hydroxytryptophan deposited gradually. The fine precipitate, which has a decomposition point of 248–249°, was filtered off and dried for analysis.

Anal. Calcd. for $C_{11}H_{12}O_3N_2$: C, 59.97; H, 5.49; N, 12.73. Found: C, 59.91; H, 5.54; N, 12.66.

The ninhydrin and other tests gave the same results as in the case of the hydrochloride salt.

Acknowledgment.—The authors wish to express their hearty thanks to Dr. David E. Green in reading the manuscript and to Dr. Bernard J. Katchman for his help in preparing the manuscript.

Summary

The synthesis of α-hydroxytryptophan is described.

OSAKA, JAPAN RECEIVED APRIL 3, 1950

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF IOWA STATE COLLEGE]

Enzymic Synthesis of Peptide Bonds. III. The Relative Effects of Some Amino Acids and their Acyl Substituents[1]

BY SIDNEY W. FOX AND HARRY WAX[2]

The comparative extents of reaction of the benzoyl derivatives of monoaminomonocarboxylic acids with aniline in the presence of papain–cysteine have been shown to be an expression of "preferential" rates of reaction.[3,4] The anilide model was introduced by Bergmann and Fraenkel–Conrat[5]; the evaluation of many conditions which influence the reaction[3,6] has made feasible a simple gravimetric method for systematic and precise comparisons of the reactivity of various substrates. Prior to application of the method to substrates involving two or more amino acid residues, it was of interest to gain some insight into how the relative reactivities of various amino acid residues may be affected by the nature of acyl substituents. In the course of such studies new information on the influence of other factors, such as *p*H, has been uncovered.

(1) Journal Paper No. J-1735 of the Iowa Agricultural Experiment Station, Ames, Iowa, Project 1111. This project is supported in large part by the National Cancer Institute of the National Institutes of Health, Public Health Service.

(2) Much of the work presented in this paper is from the Ph.D. thesis of Harry Wax, Iowa State College, 1949.

(3) Fox, Minard, Wax, Pettinga and Strifert, *Fedn. Proc.*, **8**, 198 (1949); Fox, Pettinga, Halverson and Wax, *Arch. Biochem.*, **25**, 21 (1950).

(4) The terminology of preferential hydrolysis has been recently emphasized, for a carboxypeptidase, by Kabrowska, Kazenko and Laskowski, *Science*, **110**, 95 (1949), and preferential results have been found for the previously allegedly highly specific leucine aminopeptidase by Smith and Polglase, *J. Biol. Chem.*, **180**, 1209 (1949). The fact that the term, "preference," is more apt than "specificity" is thus applicable to proteases other than papain. That specificity should not be interpreted literally has been recognized for some time, *e. g.*, Tauber has stated, "A specificity that is less than 100 per cent. is not rare in enzymology," in "Chemistry and Technology of Enzymes," 1949, p. 129, *cf.* also, *e. g.*, Stahmann, Fruton and Bergmann, *J. Biol. Chem.*, **164**, 753 (1946). In the reported conflict between the Bergmann concept of specificity of pepsin and the results of hydrolytic experiments of Bull, *Rec. Chem. Prog.*, **10**, 195 (1949), if the situation is recognized to involve preference rather than specificity, the conflict does not necessarily exist. It may also be noted that even the concept of antipodal specificity requires refinement beyond the original first approximation, as in the result of experiments with the enzymic synthesis of phenylhydrazides; Bennett and Niemann, THIS JOURNAL, **72**, 1798 (1950); Milne and Stevens, *ibid.*, **72**, 1742 (1950).

(5) Bergmann and Fraenkel-Conrat, *J. Biol. Chem.*, **119**, 707 (1937).

(6) Fox and Pettinga, *Arch. Biochem.*, **25**, 13 (1950).

The effects for four benzoylamino acids showed reactivity in the descending order of leucine, alanine, glycine and valine.[3] Three of these amino acids, leucine, valine and glycine plus one other, glutamic acid, were employed in the present investigation. The four blocking groups studied were: benzoyl, *p*-nitrobenzoyl, carbobenzoxy, and carboallyloxy. Nearly all of the compounds were previously known; few of the anilides which resulted from enzymic reaction, however, had been previously described. Since establishment of identity of these materials is prerequisite to employment of the gravimetric method used in these studies, the constants of the anilides were compiled either from accumulated quantities of product in enzyme studies or from special preparations. The constants for the various anilides are given in Table I. Of the sixteen acylamino acids submitted to reaction with aniline in the presence of papain–cysteine, only carboallyloxy-L-glutamic acid failed to deposit a precipitate, even though this was tested over the wide *p*H range and the two buffer concentrations employed for the other acylamino acid reactants.[7]

The typical dependency upon *p*H of the reactions studied is shown in Figs. 1 and 2, which also illustrate the marked activating effect of citrate buffer as previously noted with the benzoylamino acids alone, under somewhat different conditions.[6] The moderately atypical behavior of the carbobenzoxy derivatives is shown in Fig. 3.

A summary of the optimal *p*H ranges found is given in Table II. These results emphasize, in conjunction with the optima of around 6 found for aromatic amino acids,[6] the effect of substrate upon the *p*H optimum of an enzyme. The importance of substrate to *p*H optimum has long

(7) Two explanations of this non-reactivity include (1) the probability that the expected anilide is soluble (*cf.* ref. 3 for a critical discussion of the contribution of solubility), and (2) that the particular structure of carboallyloxyglutamic acid is inhibitory to enzyme–substrate interaction. Addition of the acid in other anilide syntheses gave no inhibition, and the solubility explanation seems, therefore, to be more acceptable at present, although yet other steric explanations are not excluded.